Two-Photon Antenna-Core Oxygen Probe with Enhanced Performance

SUPPORTING INFORMATION

Emmanuel Roussakis,[1,2] Joel A. Spencer,[2] Charles P. Lin,[2] and Sergei A. Vinogradov[1]∗

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104, USA
Wellman Center for Photomedicine and Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

General

All solvents and reagents were obtained from commercial sources and used as received. Free-base porphyrin **1**[44] and Gen 2 glutamic dendron[45,46] were synthesized as described previously. Thin-layer chromatography was performed on aluminum-supported silica gel plates (Aldrich). Column chromatography was performed on Selecto silica gel (Fisher). Preparative size exclusion chromatography (SEC) was performed on S-X1 beads (Bio-Rad), using tetrahydrofuran (THF) as a mobile phase. ^{1}H NMR spectra were recorded on a Bruker DPX-400 spectrometer. Mass-spectra were obtained on a MALDI-TOF Voyager-DERP Bio-Spectrometry workstation, using *alpha*-cyano-4-hydroxycinnamic acid as a matrix. Quartz fluorometric cells (Starna, Inc, 1 cm optical path length) were used in linear optical experiments. Optical spectra were recorded on a Perkin-Elmer Lambda 40 UV-Vis spectrophotometer. Steady state fluorescence and phosphorescence measurements were performed on a FSP920 spectrofluorometer (Edinburgh Instruments), equipped with a photon counting PMT R2658P PMT (Hamamatsu). For all emission measurements, the absorbance of the sample at the excitation wavelength was kept below 0.1 OD. The slits on both excitation and emission monochromators were 1 nm, and the total counts on the signal did not exceed 2×10^5 cps (counts-per-second). Emission spectra were corrected by the detector quantum efficiency response curve and by the incident light intensity at the excitation wavelength. For phosphorescence quantum yield measurements solutions were deoxygenated by Ar (Airgas, Grade 5.5). Time resolved phosphorescence measurements were performed using an in-house constructed phosphorometer.[49] Stern-Volmer oxygen quenching plots were measured using an earlier designed setup.[46] Fluorescence lifetime measurements were performed using time-correlated single photon counting method as described previously.[38]

Animal protocols

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital. A male C57BL/6 Nes-GFP mouse was anesthetized *via* inhalation of a mixture of 1.35-2% isofluorane and O_2. To maintain anesthesia the mixture was reduced to 1.25%

isofluorane with 98.75% O_2. This protocol minimizes the effect of anesthesia on tissue pO_2. Calvarial bone was exposed through a U-incision in the scalp to create a skin flap, and 2% methocellulose gel was placed on the scalp for refractive index matching. The mouse was kept on a warm stage with its skull positioned under the objective. An approximately 4x6 mm area of the calvarium encompassing most of the parasagittal bone marrow cavities within the left and right frontal bones was imaged (bone by SHG, vasculature by 2P fluorescence) and appropriate locations were selected for pO_2 measurements.

Synthesis

PtTCHP octa-butyl ester (2). Free base porphyrin **1** (215 mg, 0.162 mmol) was dissolved in benzonitrile (30 ml), and $PtCl_2$ (86 mg, 0.234 mmol) was added. The mixture was refluxed in the dark under Ar. The progress in Pt insertion was monitored by UV-Vis spectroscopy and fluorometry (until the fluorescence of free-base porphyrin disappeared). The conversion typically required 6-8 h. The solvent was evaporated under vacuum, and the remaining residue was purified on a silica gel column (CH_2Cl_2:THF, 100:1-50:1). The red band was collected and dried under vacuum, to give the product as a red solid. Yield of **2**: 225 mg (91%). ^{1}H NMR ($CDCl_3$): δ: 9.86-9.60 (m, 4H), 4.71-3.75 (m, 40H), 1.78-1.60 (m, 16H), 1.50-1.36 (m, 16H), 0.97-0.84 (m, 24H); MALDI-TOF: *m/z* 1521.8 $(M+H)^+$, calc. 1520.7; UV-vis (dichloromethane), λ_{max},nm (ε, $cm^{-1}M^{-1}$): 381 (323,000), 536 (71,000).

PtTCHP acid (3). To a solution of PtTCHP octabutyl ester (182 mg, 0.12 mmol) in a 10:1 mixture of THF/methanol (35/3.5 ml), KOH (321 mg, 5.7 mmol) and water (0.35 ml) were added. The mixture was stirred at room temperature until a red precipitate formed, leaving the mother liquor practically colorless. The heterogeneous mixture was centrifuged, the supernatant solution was decanted, and the solid material was dissolved in water (ca 10 ml). The resulting solution was acidified with an aqueous HCl until the red solid precipitated again. The material was collected by centrifugation, supernatant was removed, and the precipitate was re-dissolved in aqueous NaOH. This cycle was repeated two more times, and the final material was dried in vacuum to give **3** as a fluffy red powder. Yield: 110 mg (86%). ^{1}H NMR (DMSO-d6): δ 12.68 (br.s, 8H), 9.84-9.28 (m, 4H), 4.46-3.60 (m, 24H); MALDI-TOF: *m/z* 1072.8 $(M+H)^+$, 1111.42 $(M+K)^+$, calc. 1071.8.

2-(ethyl(6-methyl-2-oxo-4-(trifluoromethyl)-2H-chromen-7-yl)amino)acetic acid (4). Coumarin 307 (100 mg, 0.367 mmol) was dissolved in dry DMF (2.5 ml). *Tert*-butyl bromoacetate (0.24 ml, 1.61 mmol) and K_2CO_3 (456 ml, 3.3 mmol) were added, and the mixture was kept at 90^{o}C overnight. The mixture was cooled to room temperature, diluted with ethyl acetate (50 ml) and washed three times with

water and once with brine. The organic phase was collected, dried over Na_2SO_4, and the solvent was removed in vacuum. The product was purified by column chromatography (silica gel, CH_2Cl_2:CH_3OH, 10:1-2.5:1) and collected as a yellow solid. Yield: 60 mg (50%). MALDI-TOF: *m/z* 330.0 $(M+H)^+$, calc. 330.1; 284.7 $(M - CO_2)^+$, calc. 284.0.

***tert*-butyl 2-(2-(ethyl(6-methyl-2-oxo-4-(trifluoromethyl)-2H-chromen-7-yl)amino)-acetamido)-ethylcarbamate (5).** Compound **4** (50 mg, 0.15 mmol), *tert*-butyl 2-aminoethylcarbamate (27 mg, 0.17 mmol) and CDMT (29 mg, 0.17 mmol) were mixed in acetonitrile (12 ml). N-methylmorfoline (NMM) (0.033 ml, 0.30 mmol) was added to the mixture under stirring, and it was left to react overnight at room temperature. The solvent was evaporated in vacuum, the residue was dissolved in dichloromethane and the organic mixture was washed successively with water, 10% citric acid, saturated aqueous solution of $NaHCO_3$ and water, after which it was dried over Na_2SO_4. The solvent was removed by rotary evaporation, and the residue was purified on a silica gel column (eluent CH_2Cl_2:CH_3OH, 20:1-5:1) and dried under vacuum, to afford 52 mg (73%) of the product as a yellow solid. MALDI-TOF: *m/z* 470.1 $M+H^+$, calc. 471.2; 416.1 ($M+H^+$-tBu), calc. 416.0.

7-amino-6-methyl-4-(trifluoromethyl)-2H-chromen-2-one (6). 3-amino-4-methylphenol (308 mg, 2.5 mmol) was dissolved in ethanol (2.5 ml). $ZnCl_2$ (256 mg, 1.875 mmol) and ethyl trifluoroacetoacetate (0.402 mL, 2.75 mmol) were added, and the mixture was refluxed under Ar overnight. Upon cooling with an ice-water bath a yellow solid precipitated. It was collected and recrystallized from ethanol. The yellow solid was filtered and dried under vacuum, to give 450 mg (74%) of **6**. ^{1}H NMR (DMSO-d6): δ 7.21 (br. s, 1H), 6.56 (s, 1H), 6.42 (s, 1H), 6.32 (br. s, 2H), 2.12 (s, 3H).

2-(6-methyl-2-oxo-4-(trifluoromethyl)-2H-chromen-7-ylamino)acetic acid (7). Compound **6** (250 mg, 1.03 mmol) was dissolved in dry DMF (1 ml), *tert*-butyl bromoacetate (0.18 mL, 1.23 mmol) was added to the mixture, and it was kept at 80^{o}C overnight. The reaction progress was monitored by TLC (CH_2Cl_2:CH_3OH, 5:1). The mixture was diluted with ethyl acetate, washed three times with water and once with brine, dried over Na_2SO_4, filtered and the solvent was removed by rotary evaporation. The residue was purified on a silica gel column (eluent CH_2Cl_2:CH_3OH, 10:1-2.5:1), and the product was dried under vacuum to afford 200 mg (65%) of **7** as a yellow solid. ^{1}H NMR (DMSO-d6): δ 7.26 (br.s, 1H), 6.45 (s, 1H), 6.38 (s, 1H), 6.23 (br.s, 1H), 3.78 (s, 2H), 2.18 (s, 3H); MALDI-TOF: *m/z* 302.3 $(M+H)^+$, calc. 301.2.

tert-butyl 2-(2-(6-methyl-2-oxo-4-(trifluoromethyl)-2H-chromen-7-ylamino)-acetamido)ethyl-carbamate (8). Compound **7** (150 mg, 0.50 mmol) was dissolved in dry DMF (10 ml) and HBTU (208 mg, 0.55 mmol) was added in one portion. The mixture was stirred for 5 min, and N,N-diisopropylethylamine (0.13 mL, 0.75 mmol) was added, followed immediately by addition of *tert*-butyl

2-aminoethylcarbamate (88 mg, 0.55 mmol) as a solution in dry DMF (2 ml), after which the mixture was left overnight under stirring. The reaction mixture was diluted with dichloromethane and washed three times with water and once with brine, dried over Na_2SO_4, and the solvent was removed by rotary evaporation. After purification on a silica gel column (eluent CH_2Cl_2:CH_3OH, 20:1-5:1) and drying under vacuum, 8 (155 mg, 70%) was isolated as yellow solid. ^{1}H NMR (DMSO-d6): δ 8.06 (t, 1H, J=5.6 Hz), 7.27 (br.s, 1H), 6.82 (t, 1H, J=5.6 Hz), 6.56 (t, 1H, J=6 Hz), 6.50 (s, 1H), 6.33 (s, 1H), 3.83 (d, 2H, J=6Hz), 3.13-3.09 (m, 2H), 2.30-2.95 (m, 2H), 2.20 (s, 3H), 1.35 (s, 9H); UV-vis (ethanol), λ_{max}, nm (ε, $cm^{-1}M^{-1}$): 390 (18,600).

PtTCHP(EDA-C307)$_8$ (10). Boc-protection was removed from coumarin **8** prior to the reaction. For that **8** (27 mg, 0.06 mmol) was dissolved and kept in trifluoroacetic acid (TFA, 3 ml) for 2 h at room temperature. TFA was removed by rotary evaporation, dichloromethane was added to the residue and the solvent was evaporated. Addition of CH_2Cl_2 followed by rotary evaporation was repeated two more times, and the remaining solid was dried under vacuum overnight.

PtTCHP (**3**) (6.4 mg, 0.006 mmol) was dissolved in dry DMF (5 ml) and HBTU (23 mg, 0.06 mmol) was added in one portion. The mixture was stirred for 5 min, and N,N-diisopropylethylamine (0.026 mL, 0.15 mmol) was added, followed immediately by addition of a solution of the deprotected coumarin in dry DMF (2 ml). The mixture was stirred overnight at room temperature. The reaction mixture was diluted with dichloromethane and washed three times with water and once with brine, dried over Na_2SO_4, and the solvent was removed by rotary evaporation. The residue was purified by chromatography on a silica gel column (eluent CH_2Cl_2:CH_3OH, 20:1-5:1) and dried under vacuum, to afford 15 mg (68%) of **10**. MALDI-TOF: *m/z* 3692 $(M+Na)^+$, calc. 3695.

PtTCHP-octaethylbutyrate (11). PtTCHP (**3**) (100 mg, 0.09 mmol) was dissolved in dry DMF (45 mL) and HBTU (662 mg, 1.75 mmol) was added in one portion. The mixture was stirred for 5 min and N,N-diisopropylethylamine (0.68 mL, 3.88 mmol) was added, followed immediately by addition of ethyl 4-aminobutyrate·HCl (260 mg, 1.55 mmol) as a solution in dry DMF (5 ml). The mixture was left overnight under stirring. The reaction mixture was diluted with dichloromethane and washed three times with water and once with brine, dried over Na_2SO_4, and the solvent was removed by rotary evaporation. The residue was purified on a silica gel column (eluent CH_2Cl_2:THF, 20:1-10:1) and dried under vacuum, to afford 157 mg (85%) of **11** as a red solid. MALDI-TOF: *m/z* 1076.5 $(M+H)^+$, 1999.5 $(M+Na)^+$, calc. 1075.8.

PtTCHP-octabutyric acid (12). To a solution of **11** (125 mg, 0.06 mmol) in a 10:1 mixture of THF/methanol (20/2 mL), KOH (208 mg, 3.7 mmol) and water (0.2 ml) were added. The mixture was stirred at room temperature until red solid precipitated, leaving the mother liquor colorless. The solid was

separated by centrifugation, the supernatant was decanted, and the solid was dissolved in water (5 ml) and acidified with HCl aq. until the red solid precipitated again. The mixture was centrifuged, the supernatant solution was removed, and the solid was dissolved again in NaOH aq. (1N). The precipitation/centrifugation cycle was repeated again and the resulting red solid was washed with water and dried under vacuum to give **12** as a fluffy red solid. Yield: 88 mg (80%). ^{1}H NMR (DMSO-d6): δ 12.29 (br.s, 8H), 10.00 (br.s, 4H), 8.36 (br.s, 8H), 4.43-3.65 (m, 40H), 2.51-2.31 (m, 16H), 1.83-1.73 (m, 16H).

PtTCHP-Glu^{2}OEt (13). Gen 2 Boc-protected glutamic dendrom (Boc-NH-Glu^{2}OEt) was deprotected prior to the reaction. For that Boc-NH-Glu^{2}OEt (84 mg, 0.137 mmol) was dissolved in TFA and kept in that solution for 2 h at room temperature. TFA was removed by rotary evaporation, dichloromethane was added to the residue and the solvent was evaporated. Addition of CH_2Cl_2 followed by rotary evaporation was repeated two more times, and the resulting solid was dried under vacuum overnight.

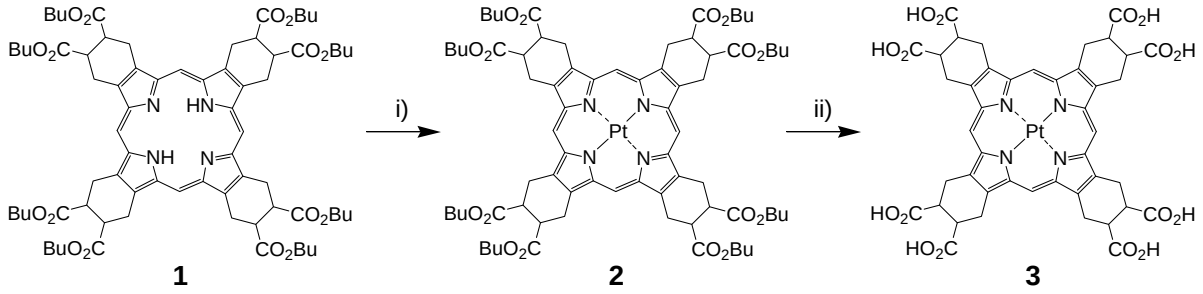
PtTCHP-octabutyric acid (**12**) (20 mg, 0.011 mmol) was dissolved in dry DMF (15 ml) and HBTU (52 mg, 0.137 mmol) was added in one portion. The mixture was stirred for 5 min and N,N-diisopropylethylamine (0.09 mL, 0.51 mmol) was added, followed immediately by addition of deprotected dendron H_2N-Glu^{2}OEt in dry DMF (5 ml). The reaction mixture was stirred overnight at room temperature and then poured into slightly acidified (pH 2-3) ice-cold water. The red residue was washed with water and dried in vacuum to afford 12 as red solid. Yield: 60 mg (92%). MALDI-TOF: *m/z* 5697.6 (center), 5092.8 (center), calc. 5749.1.

PtTCHP-Glu^{2}OH (14). PtTCHP-Glu^{2}OEt (**13**) (50 mg, 0.0087 mmol) was dissolved in a mixture of THF and MeOH (5 ml THF/0.5 ml MeOH), KOH (120 mg, 2.14 mmol), a few drops of water were added, and the mixture was stirred at room temperature for ~1 h until red solid precipitated, leaving the mother liquor practically colorless. The mixture was centrifuged and the supernatant was decanted. The remaining solid material was dissolved in water (5 ml) and re-precipitated by addition of HCl aq. The mixture was centrifuged again, the supernatant solution was removed, and the solid was re-dissolved in NaOH aq. (10 ml). The precipitation/centrifugation was repeated, and the resulting solid was dried under vacuum, to afford **12** as a red fluffy solid. Yield: 30 mg (71%). MALDI-TOF: *m/z* 4722.9 (center), calc. 4851.42.

PtTCHP-C307 (15). Boc-protected coumarin **8** (3.8 mg, 0.0086 mmol) was deprotected by TFA, dried as described above (see synthesis of **10**) and dissolved in dry DMF (2 ml). PtTCHP-Glu^{2}OH (**14**) (10 mg, 0.002 mmol) was dissolved in dry DMF (5 ml) and HBTU (31 mg, 0.082 mmol) was added in one portion. The mixture was stirred for 5 min and N,N-diisopropylethylamine (0.025 ml, 0.14 mmol) was added, followed immediately by addition of solution of deprotected **8**. The mixture was protected

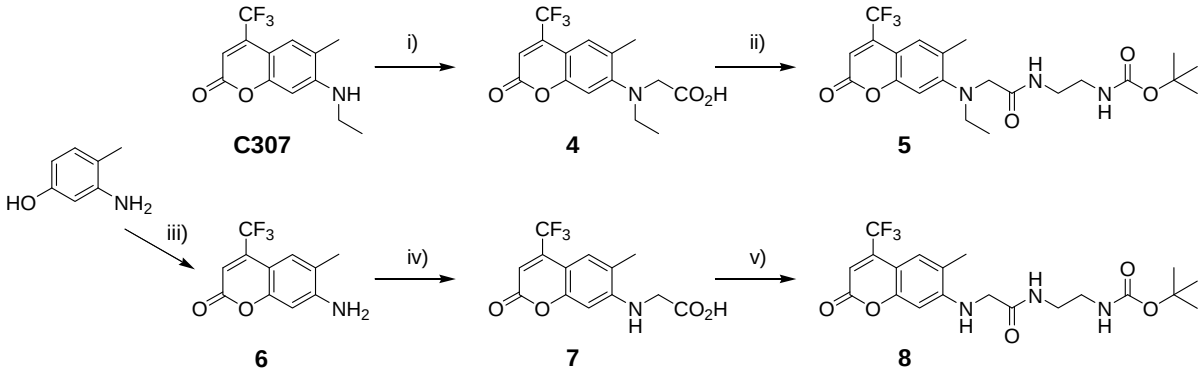
from light and stirred at room temperature for 5 h. A solution of mPEG-NH_2 (amino-polyethyleneglycol monomethylester, Av. MW 1,000) (144 mg, 0.14 mmol) in dry DMF (3 ml) was added, and the mixture was allowed to react overnight at room temperature, protected from ambient light. Excess diethyl ether was added to precipitate the product, which was collected by centrifugation. The product was dissolved in a small volume of THF, re-precipitated upon addition of excess ether, and centrifuged again. This washing procedure was repeated twice to remove most of the excess mPEG-NH_2. The product was subsequently dissolved in THF and purified by size-exclusion chromatography using THF as eluent. The red band was collected and the solvent was removed by rotary evaporation. The remaining material was dissolved in water, passed through a 20 μm filter, and freeze dried to afford 48 mg (~85%) of the two-photon probe as a red solid. MALDI-TOF: *m/z* 27866 (center), calc. ~33,000.

Scheme S1



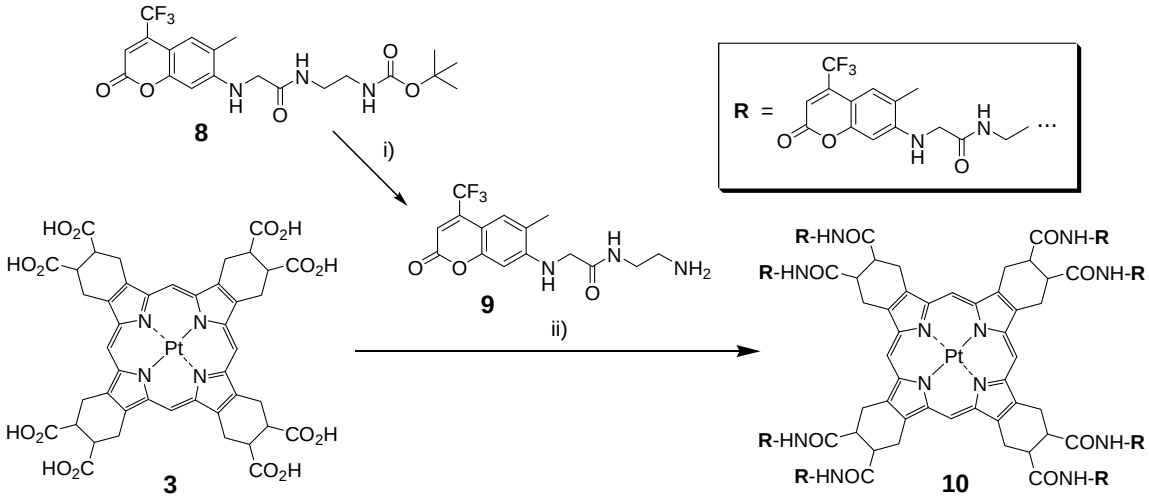
Reagents and conditions: i) $PtCl_2$, benzonitrile, reflux, 6-8 h (91%); ii) KOH, THF/CH_3OH/H_2O, rt (86%).

Scheme S2



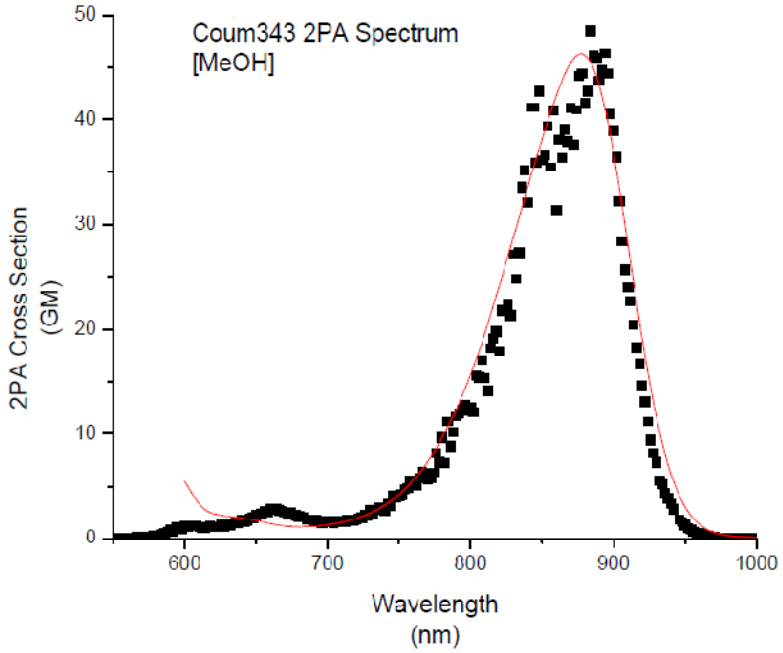
Reagents and conditions: i) $BrCH_2CO_2tBu$, K_2CO_3, DMF, 90°C, overnight (50%); ii) $H_2N(CH_2)_2NHCO_2tBu$, CDMT/N-methylmorpholine, CH_3CN, rt, overnight (73%); iii) $ZnCl_2$, $CF_3COCH_2CO_2CH_2CH_3$, ethanol, reflux, overnight (74%); iv) $BrCH_2CO_2tBu$, DMF, 80°C, overnight (65%); v) $H_2N(CH_2)_2NHCO_2tBu$, HBTU/ DIPEA, DMF, rt, overnight (70%).

Scheme S3

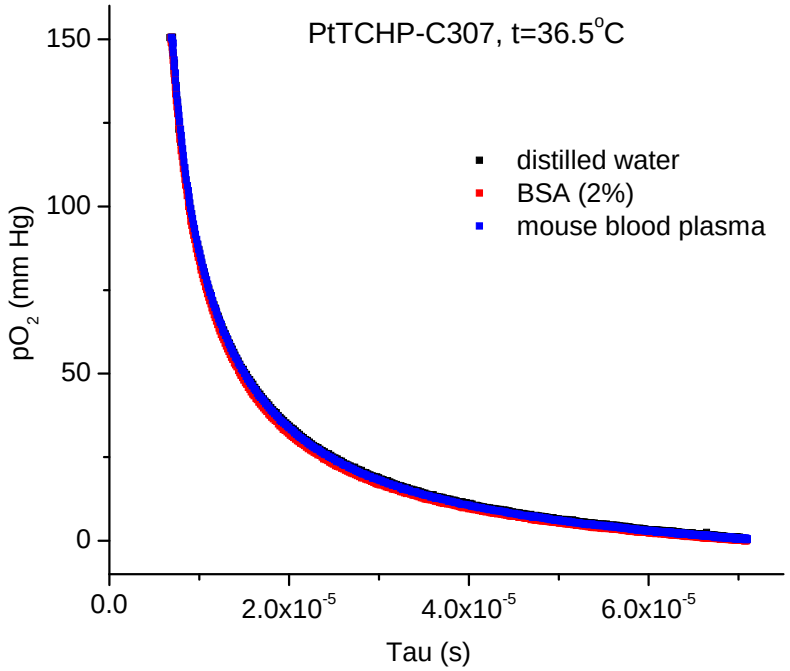


Reagents and conditions: i) TFA, rt, 2 h; ii) 9, HBTU, DIPEA, DMF, rt, overnight (68%).

2PA spectrum of Coumarin 343

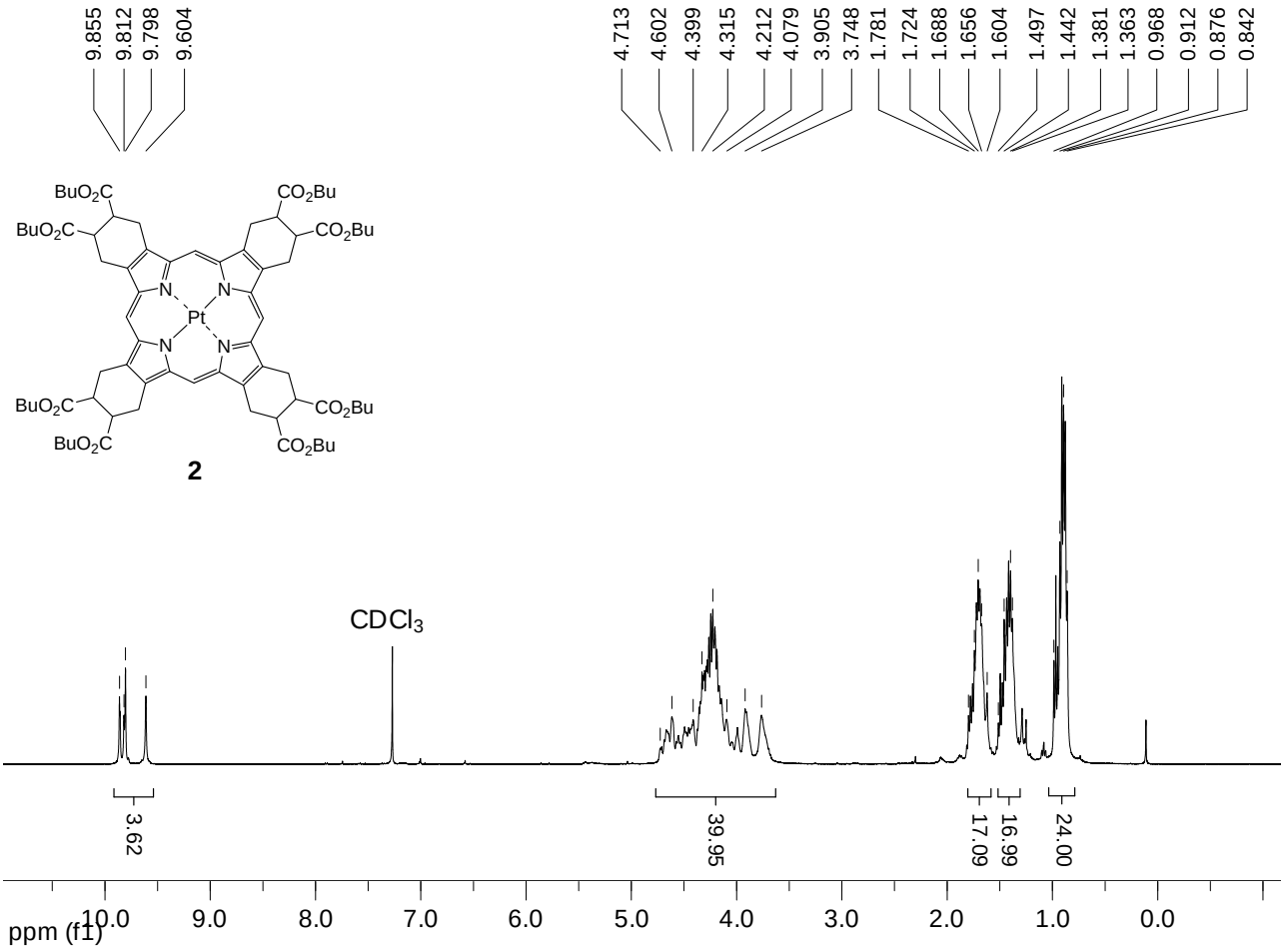


Oxygen titration plots of PtTCHP-C307 in distilled water, 2% solution of BSA and mouse blood plasma at 36.5^{o}C. A drop of heparin was added to the plasma to prevent coagulation under stirring.



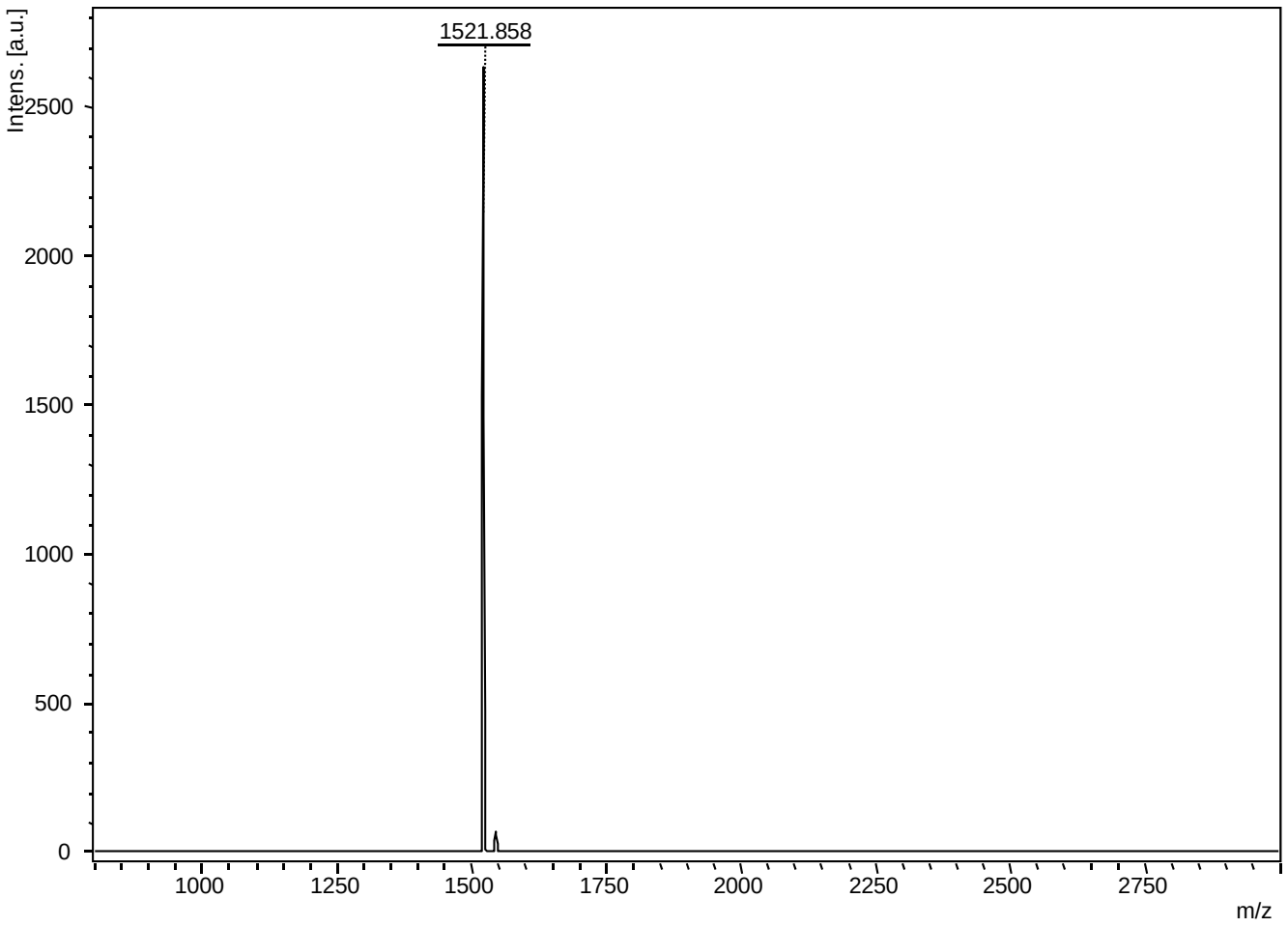
Compound **2**

^{1}H NMR



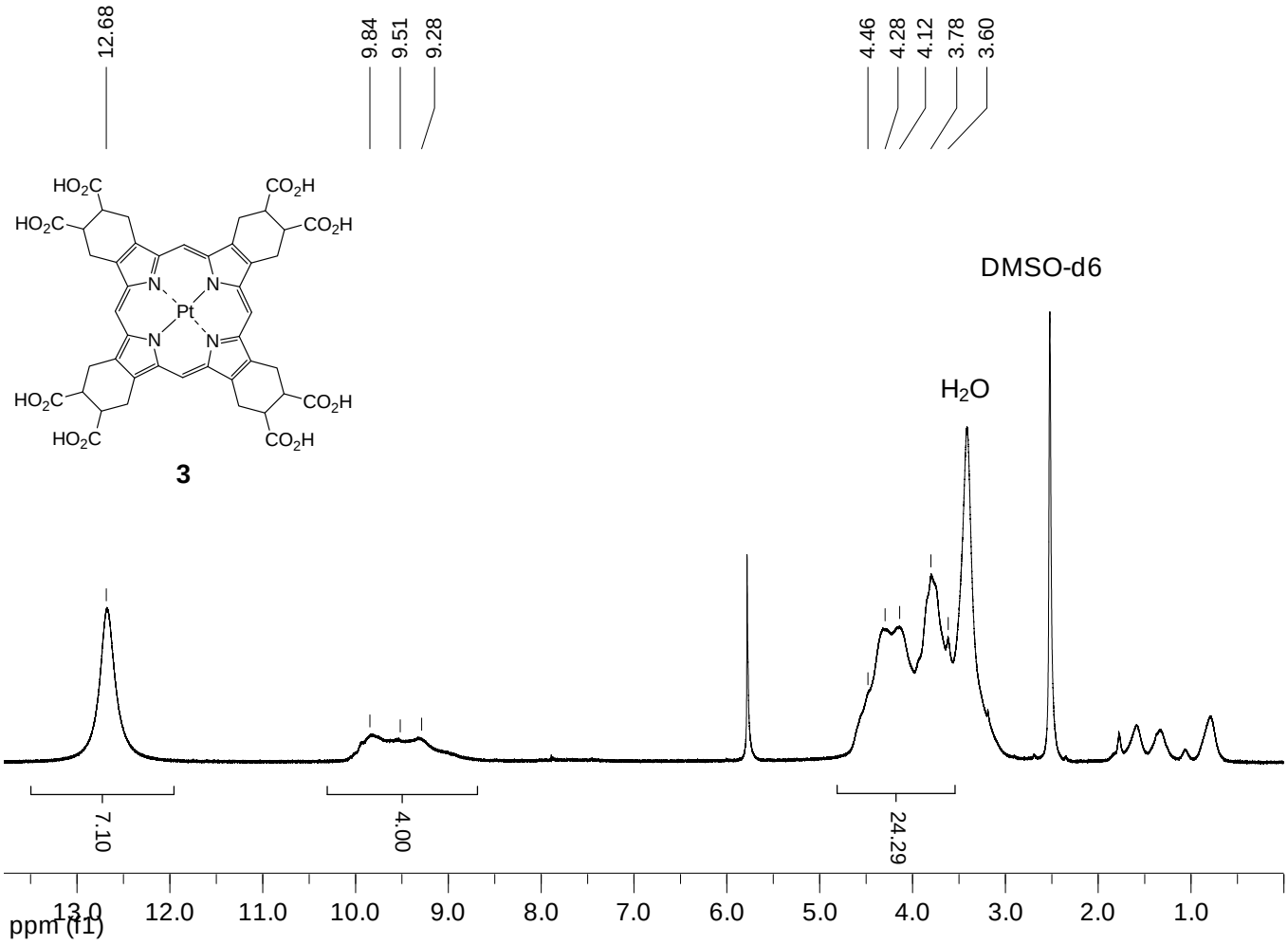
Compound **2**

MALDI-TOF



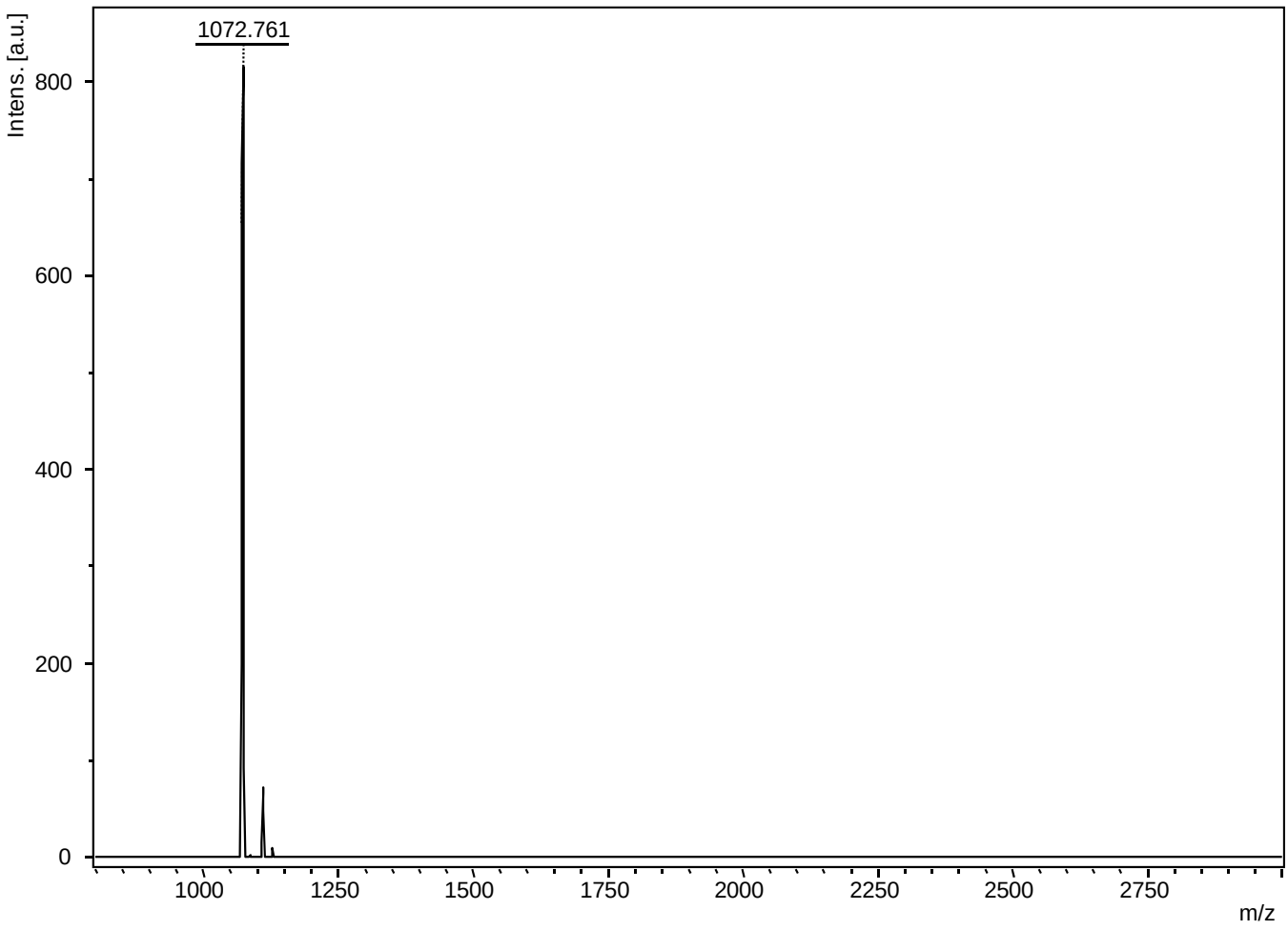
Compound **3**

^{1}H NMR



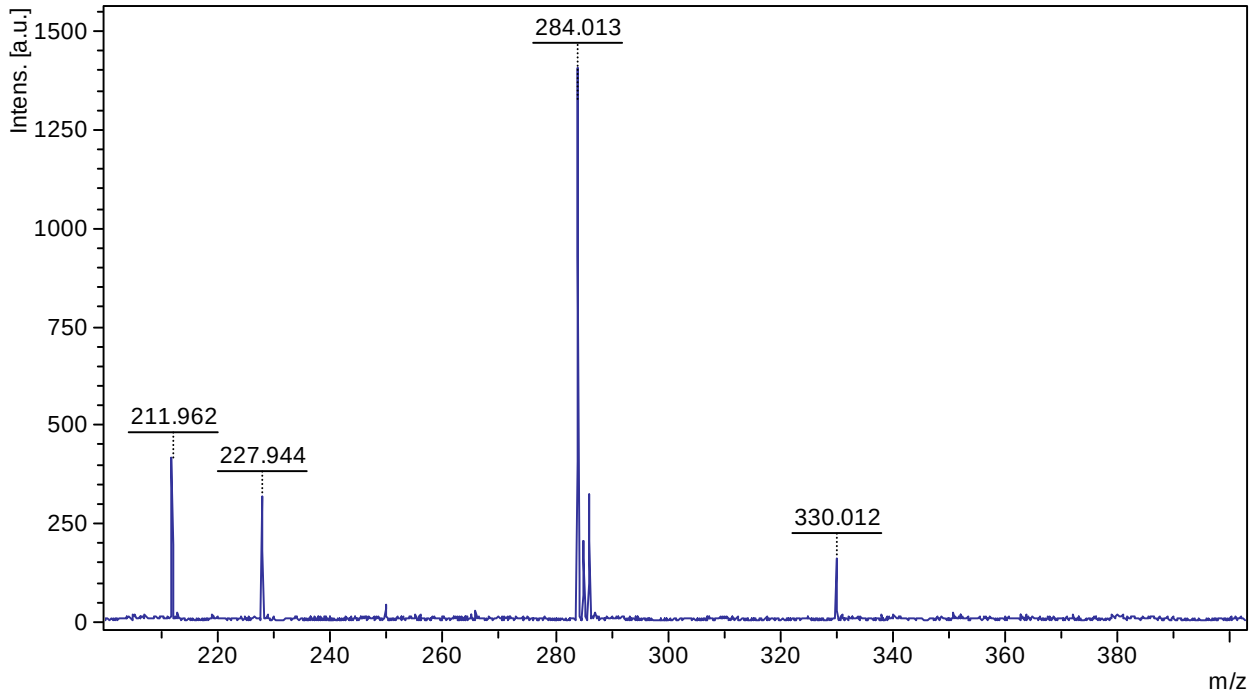
Compound **3**

MALDI-TOF



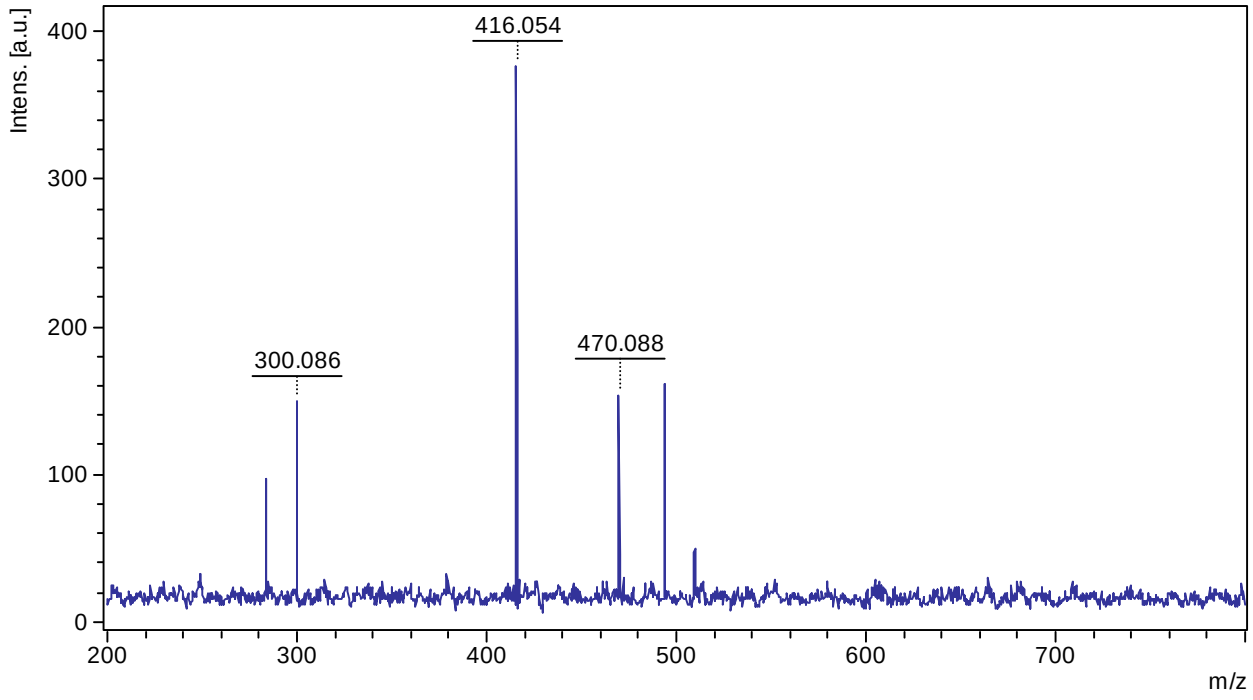
Compound **4**

MALDI-TOF



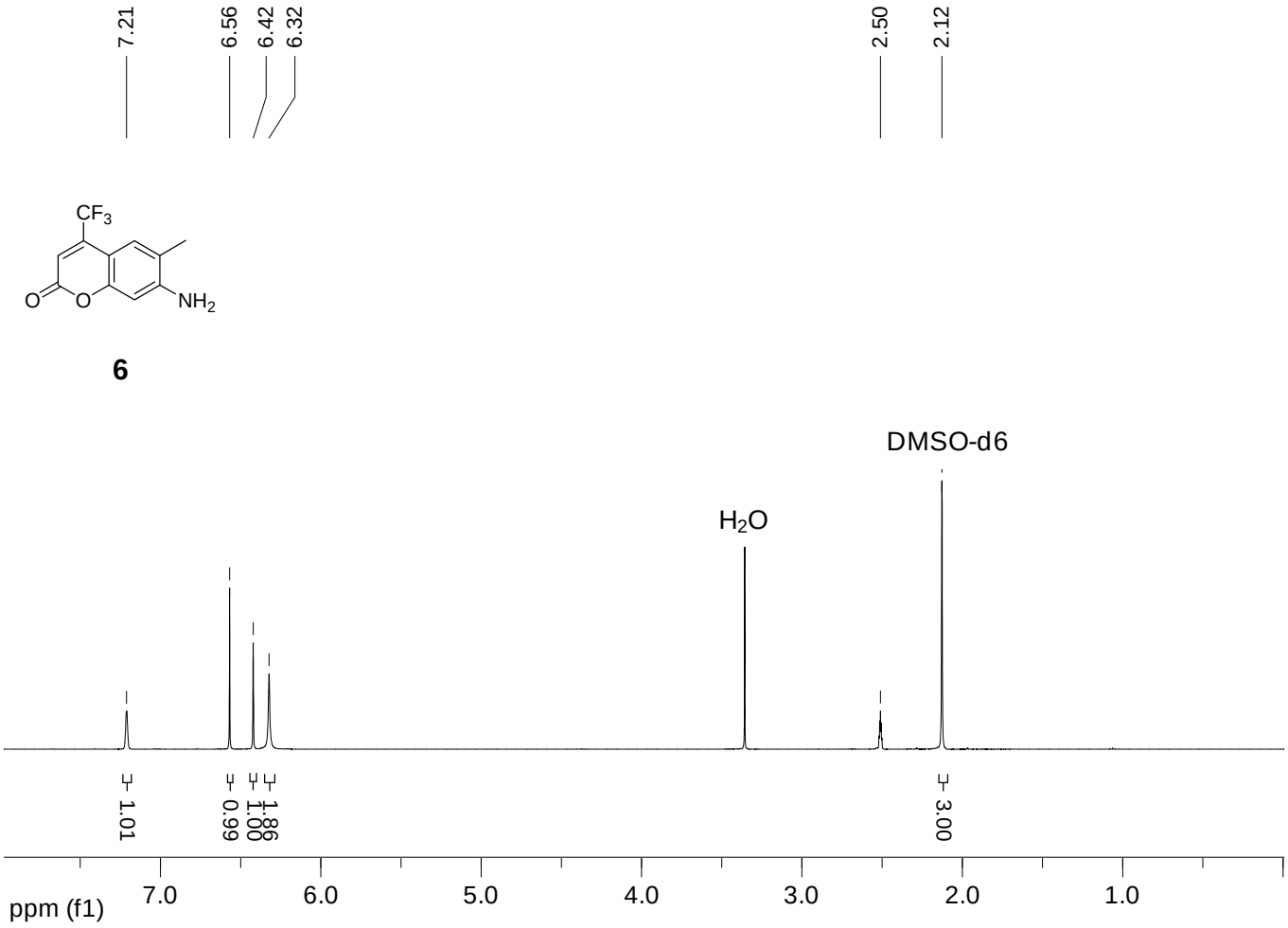
Compound **5**

MALDI-TOF



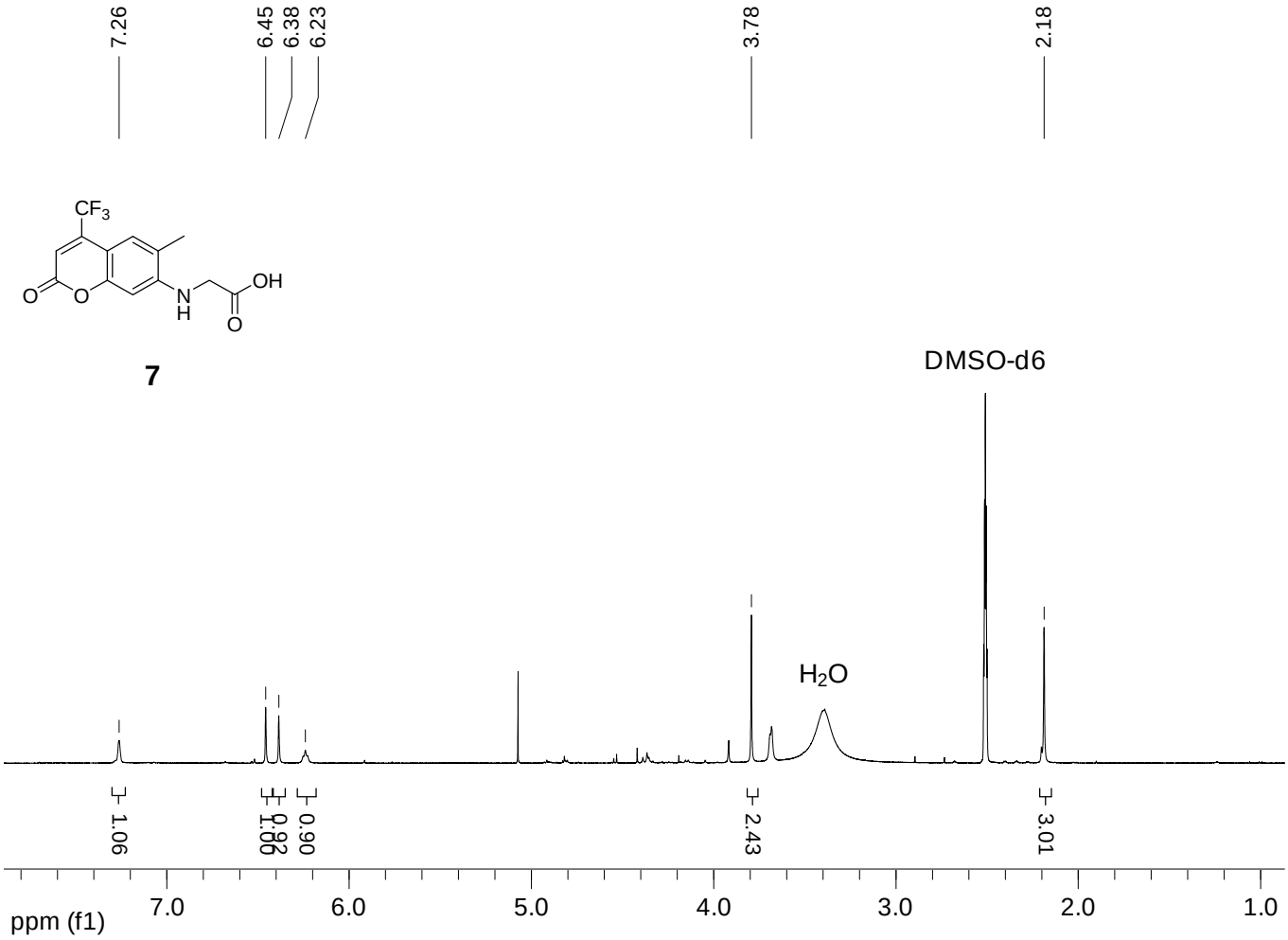
Compound **6**

^{1}H NMR



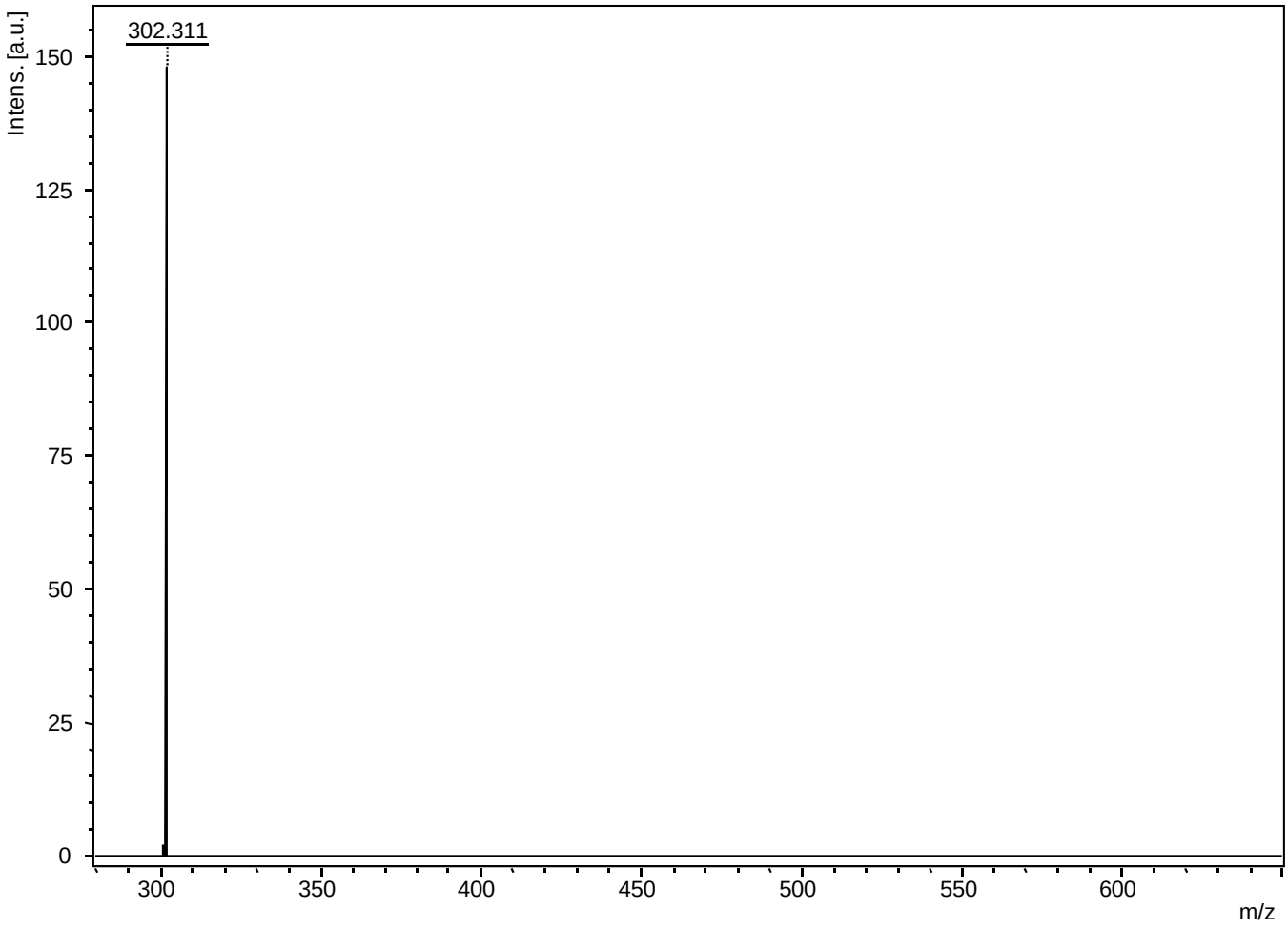
Compound **7**

^{1}H NMR



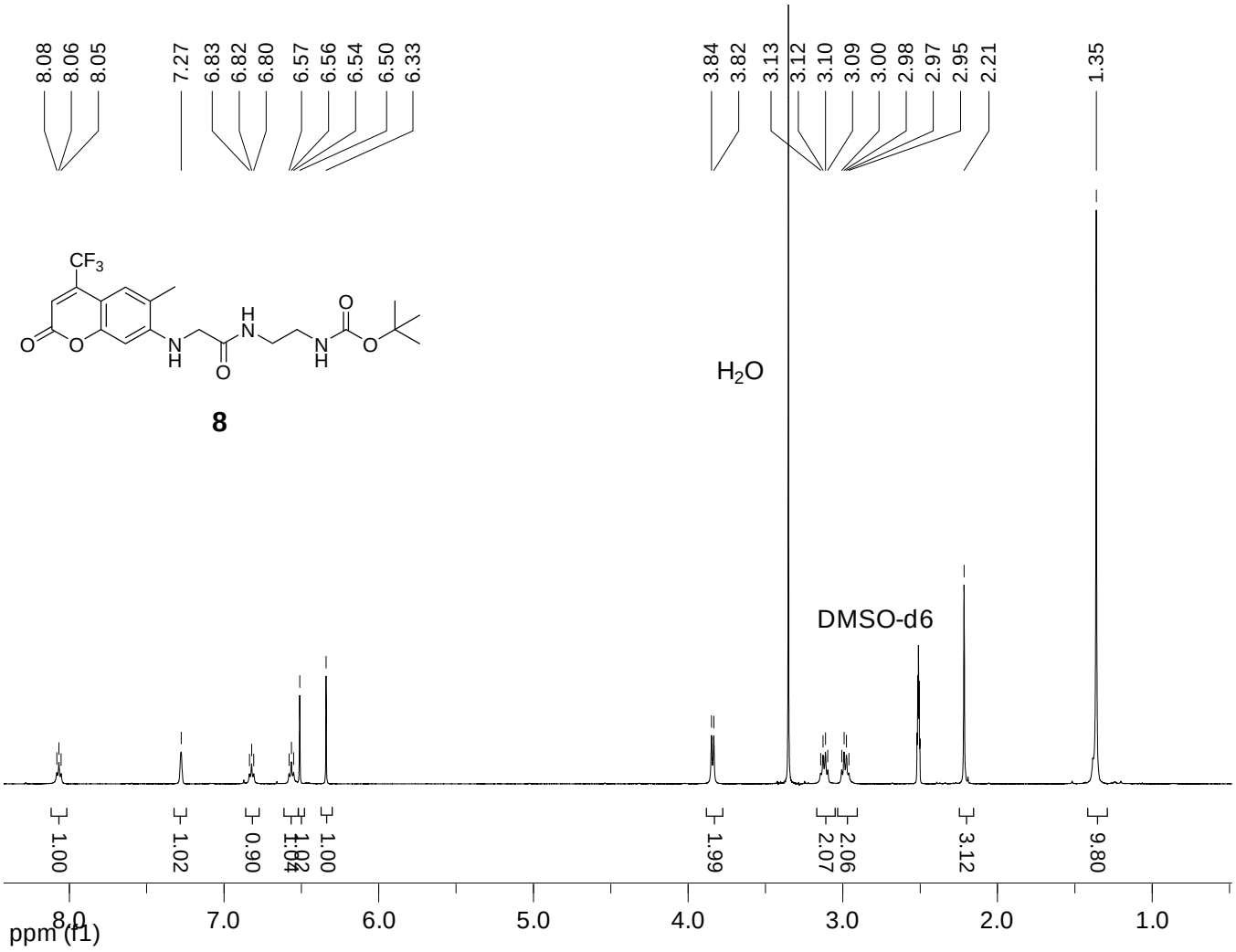
Compound **7**

MALDI-TOF



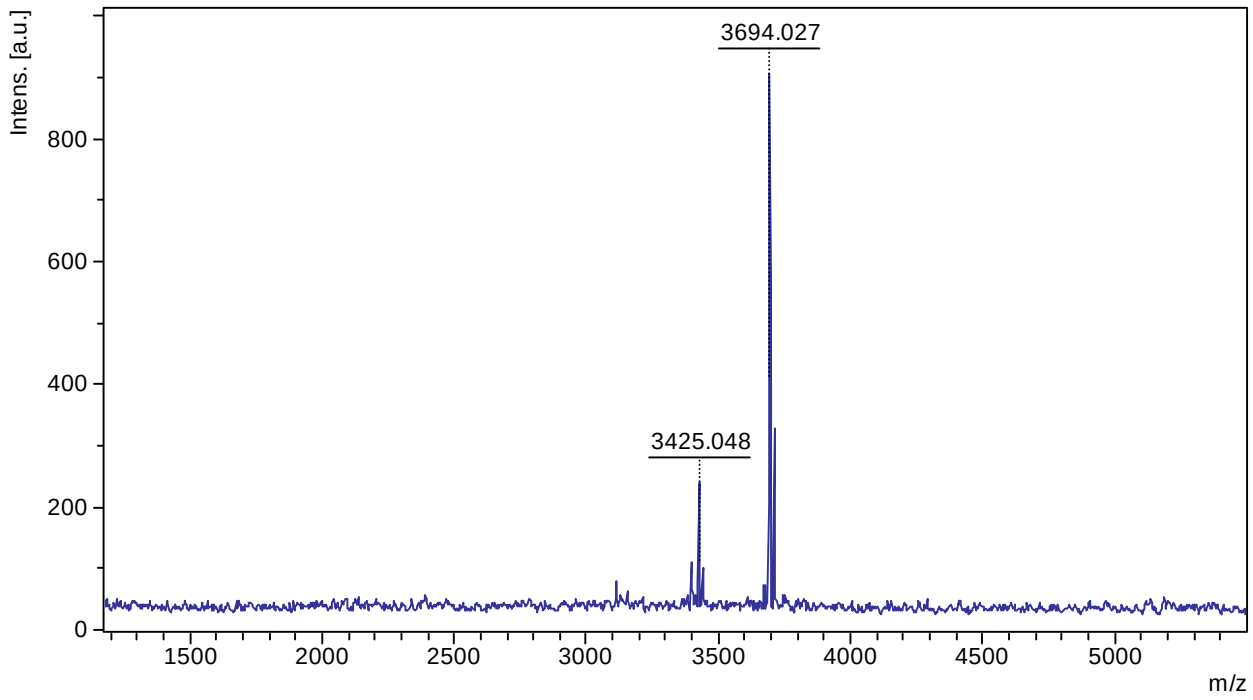
Compound **8**

^{1}H NMR



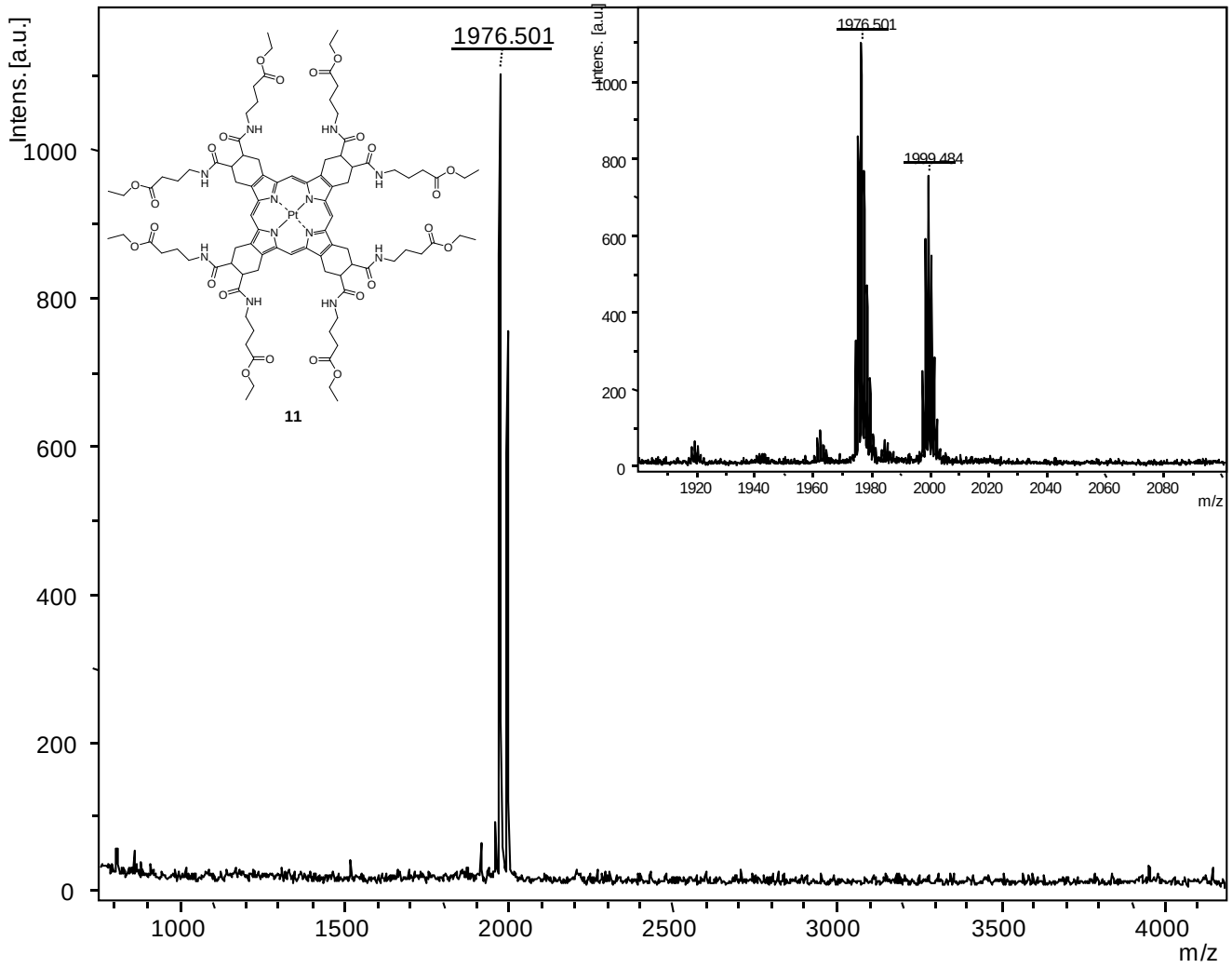
Compound **10**

MALDI-TOF



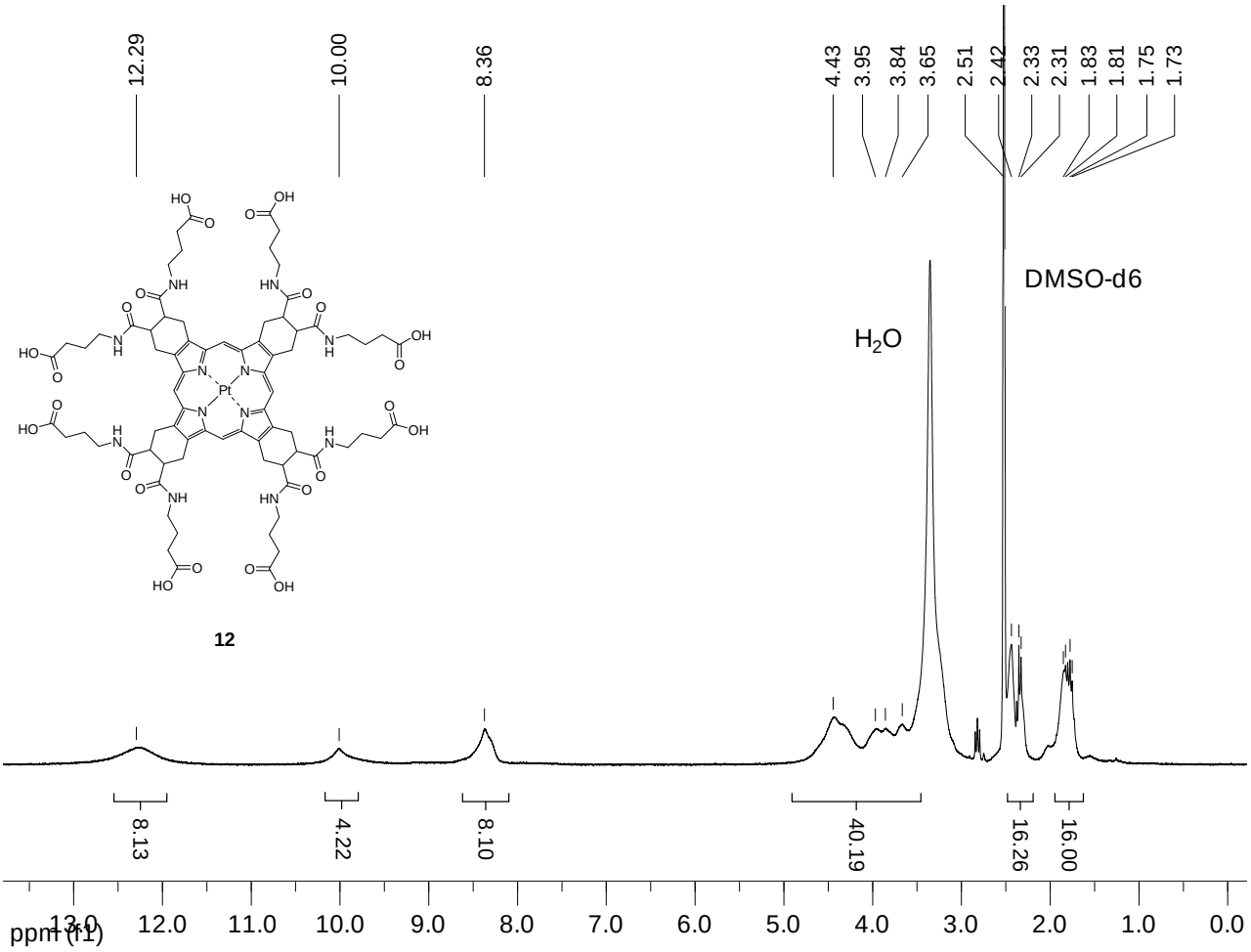
Compound **11**

MALDI-TOF



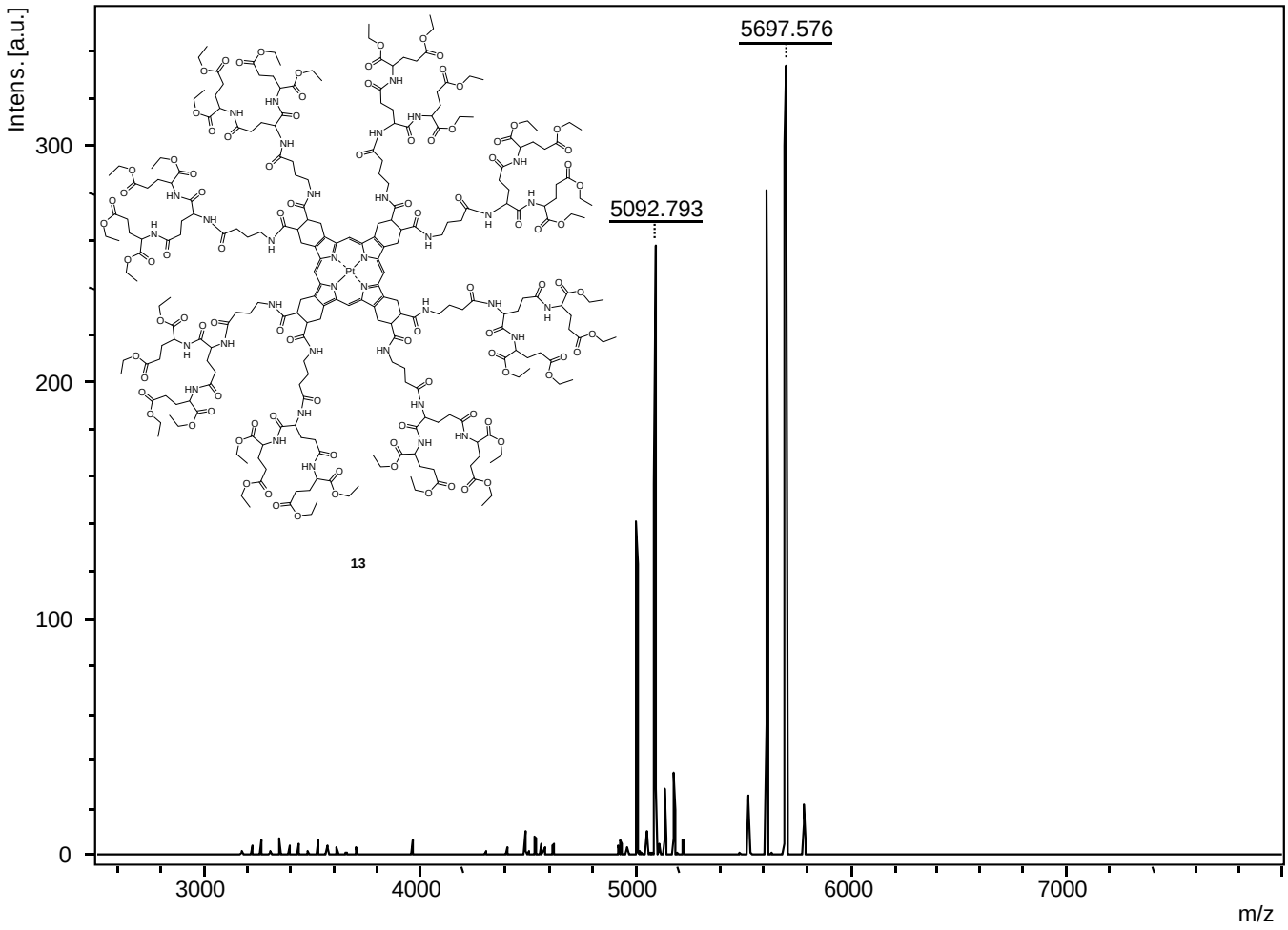
Compound **12**

^{1}H NMR



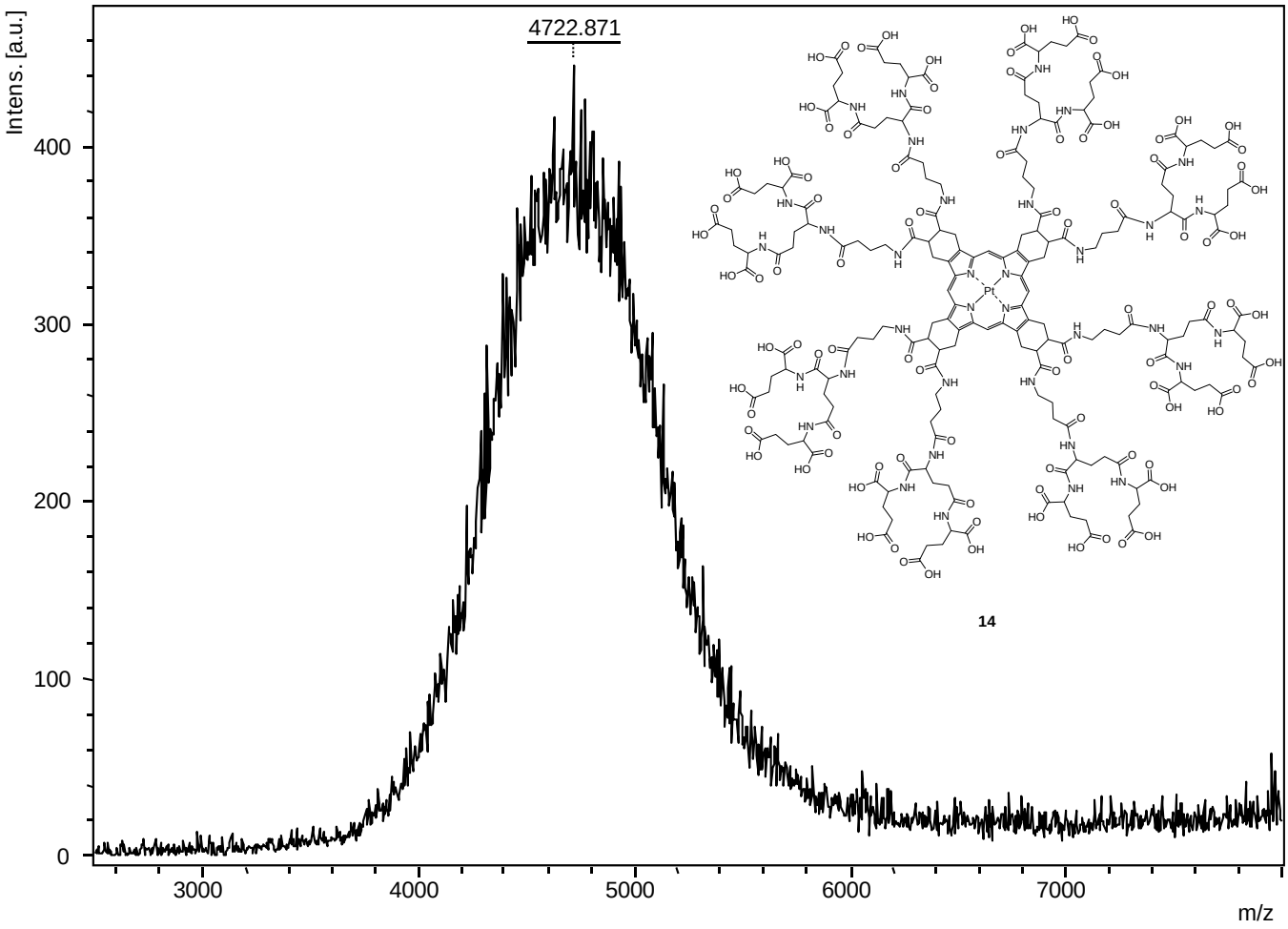
Compound **13**

MALDI-TOF



Compound **14**

MALDI-TOF



Compound **15**

MALDI-TOF